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Improved Breadth and Potency of an HIV-1-Neutralizing Human Single-chain Antibody by Random Mutagenesis and Sequential Antigen Panning

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Several human monoclonal antibodies can neutralize a range of human immunodeficiency virus type 1 (HIV-1) primary isolates but their potency and related ability to suppress generation of HIV-1 escape mutants is significantly lower than the activity of antiretroviral drugs currently in clinical use. Recently, a human Fab, X5, was identified and found to neutralize primary isolates from different clades. Further improvement of the potency and breadth of HIV-1 neutralization by this antibody could be critical for its potential use in the treatment of HIV-1-infected patients. However, increasing potency of an antibody by selection from libraries may lead to a decrease in the breadth of neutralization. In an attempt to solve this problem, we subjected a random mutagenesis library of the scFv X5 to sequential rounds of selection on non-homologous HIV-1 envelope glycoproteins (Envs) dubbed sequential antigen panning (SAP). By using SAP, we identified two scFv antibodies, m6 and m9, that were tested with a panel of 33 diverse primary HIV-1 infectious isolates in an assay based on a reporter cell-line expressing high levels of CD4, CCR5 and CXCR4. The IC_{50} was less than 50 μg/ml for 21 (m6) and 19 (m9) out of 29 isolates from group M (subtypes A–C, F, G and CRF-01AE) and one isolate from group N; three isolates from group O were not significantly inhibited at 50 μg/ml. The average IC_{50} values for the two antibodies were significantly ($p < 0.001$, $n = 29$) lower compared to scFv X5. Their inhibitory activity does not appear to be related to the HIV-1 subtype, coreceptor usage or the disease stage. m9 inhibited infection of peripheral blood mononuclear cells by the primary isolates JRCSF, 89.6 and BR020 with IC_{90} of 4, 6 and 25 μg/ml, respectively; for a single-round infection by pseudovirus, the IC_{90} for JRSCF, 89.6, YU2 and HXBc2 was 15, 5, 15 and 5 μg/ml, respectively. In these two assays the IC_{90} for m9 was, on average, two- to threefold lower than for scFv X5. These results demonstrate that both the potency and the breadth of HIV-1 neutralization of one of the few known potent broadly cross-reactive human monoclonal antibodies, scFv X5, could be improved significantly. However, only experiments in animal models and clinical trials in humans will show whether these new scFvs and the approach for their identification have potential in the development of prophylactics and therapeutics for HIV-1 infections.

Keywords: HIV; antibody; phage display; gp140; inhibitors

Abbreviations used: hmAb, human monoclonal antibody; SAP, sequential antigen panning; SOE, splice overlap extension; VH, variable region of the heavy chain; VL, variable region of the light chain; sCD4, two-domain soluble CD4; gp, gene product; PBMC, peripheral blood mononuclear cell; HRP, horse radish peroxidase.

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Introduction

Major problems in the current treatments of human immunodeficiency virus type 1 (HIV-1) infections are the ability of the virus to rapidly generate mutants resistant to drugs and the side-effects of the drug regimens employed. Several human monoclonal antibodies (hmAbs) exhibit potent and broad HIV-1 neutralizing activity *in vitro* and can prevent HIV-1 infection in animal models.[1–3] A recent clinical trial suggested that two of these broadly HIV-1-neutralizing hmAbs, 2F5 and 2G12, are without measurable side-effect in humans.[4,5] However, the potency of 2F5 and 2G12 used in combination in this clinical trial was significantly lower than currently used HAART regimens, and relapses did occur.[5] Further increases in the potency of the currently available broadly HIV-1-neutralizing hmAbs or development of new neutralizing hmAbs might lead to better approaches for prevention and treatment of HIV-1 infection. However, attempts to increase potency may lead to loss of breadth of neutralization.

We have developed a methodology for enhanced selection of broadly cross-reactive antibodies from phage display libraries, termed sequential antigen panning (SAP) that is based on sequentially changing the antigen in subsequent rounds of panning. SAP leads to selection of antibodies that bind only epitopes conserved among the isolates used for panning and screening; this methodology was used for selection of novel HIV-1-neutralizing hmAbs that exhibited activity against a variety of primary HIV-1 isolates.[6,7] We hypothesized that using this methodology in conjunction with random mutagenesis of known potent and broadly neutralizing antibodies could help in solving the fundamental problem of loss of breadth with an increase in potency.

Recently, we identified and characterized a broadly cross-reactive HIV-1-neutralizing hmAb Fab, X5,[8] that inhibited HIV-1 entry and Env-mediated cell fusion of more than 30 primary isolates with potency comparable to that of the well-characterized and potent broadly cross-reactive HIV-1-neutralizing hmAb IgG1 b12.[9,10] Fab X5 binds better to gp120–CD4 complexes than to gp120 alone, and the concentration required for 90% inhibition (IC_{90}) does not vary as much as that of IgG1 b12 for the HIV-1 isolates tested in our assays.[8] One caveat, however, is that for some isolates IgG1 X5 is not as potent as Fab X5, e.g. for three out of eight tested isolates the IC_{50} for IgG1 X5 was higher than for Fab X5, and for four isolates, higher than IC_{50} for scFv X5; on average, scFv X5 was more potent than Fab and IgG1 X5.[11]

To increase the potency and breadth of HIV-1 neutralization by X5, we used random mutagenesis in combination with SAP. We selected two new single-chain antibodies (scFv), designated m6 and m9, which exhibited significantly higher and broader neutralization activity than scFv X5. These antibodies could serve as leads for the development of adjuncts to current HIV-1 therapies, as microbicides for prevention of HIV-1 infection, as well as tools for dissecting mechanisms of HIV-1 entry.

Results

Generation and characterization of a scFv X5 mutant library

ScFv X5 was engineered from the Fab X5 construct by splice overlap extension (SOE)-PCR; the variable regions of both the heavy chain (VH) and the light chain (VL) were cloned into the pComb3X vector and joined by a flexible linker, $(G_4S)_3$. Point mutations were introduced into the scFv X5 construct by random mutagenesis and checked by DNA sequence analysis of 31 randomly selected clones. The mutations were distributed randomly throughout the scFv gene and the mutation frequency ranged from three to ten (average six) bases per kilobase of DNA.

Selection of m6 and m9 by sequential antigen panning (SAP)

We hypothesized that by sequentially changing antigens during panning of phage display libraries and screening the enriched libraries using different antigens, the selected phage will display scFvs against conserved epitopes shared among the respective antigens. X5 was selected by panning against the JR-FL (R5) isolate gp120 complexes; so complexes of two other recombinant soluble Envs, oligomeric $gp140_{89.6}$ (R5X4) and $gp140_{IIIB}$ (X4), with two-domain soluble CD4 (sCD4) were used as antigens for phage library panning. Screening of individual phage clones after panning was performed in phage ELISA with $gp140_{89.6}$, $gp120_{JR\text{-}FL}$, $gp140_{IIIB}$, and their complexes with sCD4. Two phage clones, designated m6 and m9, bound significantly to all antigens and were selected for further characterization. m6 was selected after the third round of panning and m9 after the fourth one. Both scFv mutants and parental scFv X5 were produced in *Escherichia coli*, purified and tested for their neutralizing activity.

Potent neutralizing activity of m6 and m9 against selected isolates

For a comparative analysis of the potency (concentrations required to achieve 50% (IC_{50}) and 90% (IC_{90}) inhibition) of m6, m9 and scFv X5 for some commonly studied isolates, we used assays based on infection of PBMCs and on a pseudovirus system. In both assays, m9 exhibited the highest potency, with IC_{90}s ranging from 4 μg/ml to 25 μg/ml, which was, on average, two- to three-fold lower than the IC_{90} for scFv X5; the inhibitory activity of m6 was, on average, comparable to that

of scFv X5 in these assays (Table 1). Note that for all tested isolates in the PBMC assay, scFv X5 exhibited higher potency than Fab X5 and IgG1 X5 as has been demonstrated recently for other isolates;[12] further, we will compare only scFv X5 with m6 and m9.

Because m6 and m9 are antibodies whose binding to gp120 is enhanced by CD4 binding (CD4i antibodies) (see below), we tested their activity in an assay where cell fusion is induced by a soluble CD4 (sCD4).[13] As expected, the activity of m6 and m9 (against HXBc2 Env) was very high, with IC_{50} of 0.01 μg/ml (data not shown). This suggests that combination of these antibodies with sCD4 or fusion proteins with sCD4 could have even higher neutralizing activity than the antibodies alone, as was shown recently for another CD4i antibody, 17b.[14]

Potent neutralization of diverse HIV-1 primary isolates

To determine whether the increase in potency against selected isolates leads to any loss of breadth of neutralization, we used a panel of primary isolates representing different subtypes. A total of 29 HIV-1 group M isolates including ten subtype B, five subtype A, three subtype C, six CRF-01_AE, three subtype F, two subtype G, one group N (YBF-30), and three group O isolates were tested (Table 2). The neutralization assay is based on a reporter cell-line expressing high levels of CD4, CXCR4 and CCR5 that may resemble some activated primary CD4 T-cells that express high levels of CCR5[15] and could be difficult to neutralize.[16] m6 inhibited 21 and m9 inhibited 19 out of 30 primary HIV-1 group M and N infectious isolates with IC_{50} of less than 50 μg/ml; the three group O isolates were not neutralized (inhibitory activity less than 10%, which is in the range of the experimental error) at 50 μg/ml, except one isolate by m6 (Table 2). Both antibodies neutralized this panel of diverse isolates significantly ($p < 0.001$, $n = 29$) better than scFv X5 (Table 2). The inhibitory activity was not dependent on the HIV-1 subtype. m6 and m9 were highly effective against most of the tested isolates from clades C and AE (Table 2); in contrast, another potent, broadly neutralizing human monoclonal antibody, 2G12, was not inhibitory for these isolates (data not shown). The neutralization conferred by m6 or m9 was independent of coreceptor usage, as both R5 and R5X4 isolates could be neutralized. Further, primary isolates derived from two subtype B-infected patients either early during the infection (5084/5-83 and 5048/7-82 both with R5 coreceptor use) or late in disease (5084/10-86 and 5048/3-91 both with adaptation to X4 usage) were neutralized equally by m6 and m9 (Table 2), suggesting that

Table 1. Neutralizing activity for selected HIV-1 isolates

A. *Neutralizing activity (IC_{90}; μg/ml) of m6, m9 and X5 for selected HIV-1 isolates measured by a PBMC-based assay*

Isolate/Ab	IgG1 X5	Fab X5	scFv X5	m6	m9
89.6	>300	>100	12	18	6
93BR020	>300	>100	>50	>50	25
JRCSF	>300	100	12	8	4

B. *Neutralizing activity (IC_{50} and IC_{90}; μg/ml) of m6, m9 and scFv X5 for selected HIV-1 isolates measured by an assay based on pseudovirus*

	M6		M9		scFv X5	
Virus	IC_{50}	IC_{90}	IC_{50}	IC_{90}	IC_{50}	IC_{90}
HXBc2	1	6	1	5	1	7
JRCSF	5	30	2.5	15	5	30
YU2	3	100	2	15	25	70
89.6	1	7	0.5	5	1	8

Table 2. Neutralization of diverse HIV-1 isolates by m6, m9 and scFv X5 at 50 μg/ml

			Inhibition (%)		
Virus[a]	Subtype gag/env	Coreceptor	m6	m9	scFv X5
92US714	/B	R5	94	94	60
92US727	/B	R5	22	24	73
92BR023	C/B	R5	82	100	57
92HT593	/B	R5X4	85	88	76
93US151	/B	R5X4	100	100	69
92US076	/B	R5X4	47	52	28
92UG031	A/A	R5	76	79	29
92UG037	A/A	R5	12	44	65
97USSN54	A/A	R5	11	11	6
92RW024	D/A	R5	45	74	42
92RW009	C/A	R5X4	81	95	57
97ZA003	C/C	R5	67	72	25(66)
98CN006	C/C	R5	92	96	34(55)
98IN017	/C	X4	91	93	30(44)
92TH001	A/E	R5	18	31	58
93TH073	A/E	R5	46	31	23(36)
93TH060	A/E	R5	58	40	48(45)
HM16	A/E	X4	100	96	nd
HM14	A/E	X4	81	45	52
CMU 08	A/E	R5X4	98	100	35
93BR019	/BF	R5	50	29	66
93BR029	B/F	R5	58	45	30
93BR020	F/F	R5X4	39	19	25
JV1083	/G	R5	29	45	22
HIV-G3	/G	R5	53	63	14
YBF-30	Grp N	R5	71	85	14
BCF-01	Grp O	R5	10	0	0
BCF-02	Grp O	R5	32	7	46
BCF-03	Grp O	R5	0	0	3
5084/5-83[b]	B	R5	70	85	53
5084/10-86AIDS[b]	B	R5X4	71	87	18
5048/7-82[b]	B	R5	51	83	0
5048/3-91AIDS[b]	B	R5X4	96	100	45

nd, not done. The values in the Table are the average of two different experiments each performed in triplicate. The standard deviation of the replicated values was, on average, 10%; 0 indicates either 0 or less than zero within the range of the standard deviation. The numbers in parentheses indicate percentage inhibition at 100 μg/ml.

[a] HIV-1 isolates. Their subtypes were determined on the basis of the envelope region and coreceptor usage using GHOST cell lines.

[b] Virus isolated from rapid progressor (5084) or late progressor (5048) with dates of isolations and disease status stated. Both patients had switch in their coreceptor usage over time.

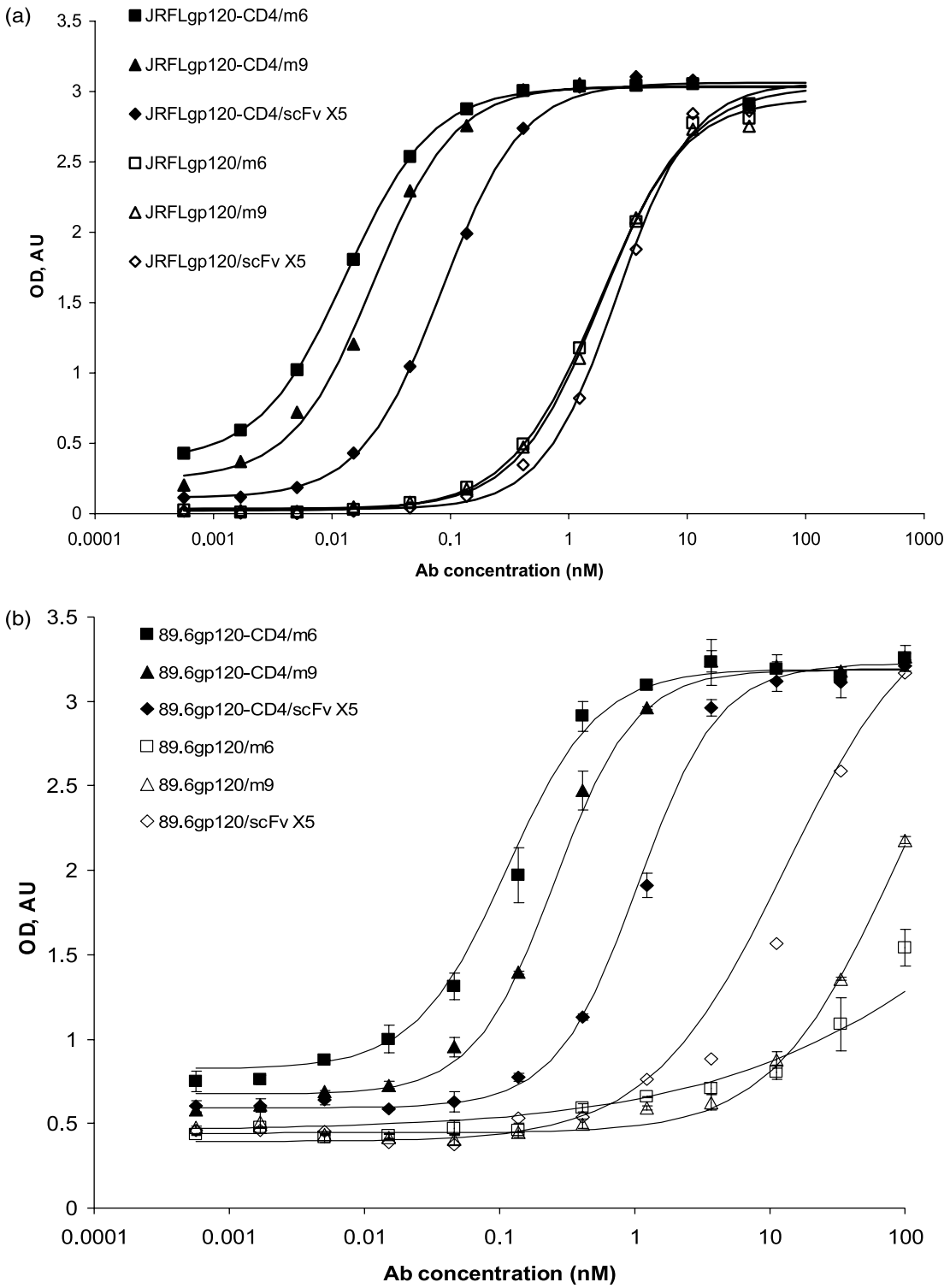

Figure 1. Binding isotherms of m6, m9 and scFv X5 to gp120 and gp120–sCD4. (a) JRFL; and (b) 89.6. gp120 and gp120–sCD4 complexes were coated directly onto 96 well plates, washed and biotinylated antibodies were added at the indicated concentrations. Bound antibodies were detected by HRP-streptavidin and measured as optical absorbance at 405 nm (A_{405}). The background was estimated by the amount of antibodies bound to BSA and subtracted. The continuous lines represent fitting of the data to the function:

$$(A - A_b)/A_{max} = C^n/(EC_{50}^n + C^n)$$

where A is optical absorbance, A_b is background absorbance, A_{max} is its maximal value, C is bulk concentration of the inhibitor, n is a constant equal to ~ 1, and EC_{50} is the concentration corresponding to half-maximal binding in the ELISA assay.

disease stage may not have an impact on the neutralizing capacity of m6 and m9. These results suggest that the improvement of the neutralizing potency of m6 and m9 is not associated with a decrease in the breadth of HIV-1 neutralization.

Increased binding to complexes of gp120 with sCD4

To find whether m6 and m9 retained the X5 property as a CD4i antibody, we measured by ELISA their binding to $gp120_{JR\text{-}FL}$ and its complex with sCD4. Both antibodies bound with high apparent affinity (EC_{50} 1.8 nM) that was increased significantly (about 100-fold) after binding of sCD4 to gp120 (0.013 nM and 0.022 nM for m6 and m9, respectively) (Figure 1(a)). These affinities are several-fold higher than the scFv X5 affinity to gp120 from the same isolate (2.5 nM) and its complex with sCD4 (0.085 nM) (Figure 1). m6 and m9 showed several-fold higher affinity for gp120 from the primary isolate 89.6 in complex with sCD4 (Figure 1(b)).

Characterization of the antibody epitopes

To begin to elucidate the mechanisms underlying the increased breadth of HIV-1 neutralization by m6 and m9 compared to scFv X5, we used two approaches for characterization of their epitopes; evaluation of their competition with anti-gp120 antibodies, and alanine scanning mutagenesis. m6 and m9 competed significantly with X5 and 17b for binding to $gp120_{89.6}$ and its complex with sCD4 (data not shown). These results suggest that the epitopes of m6 and m9 overlap the X5 and 17b epitopes and are likely located in close proximity to but outside the CD4-binding site.

To further characterize the m6 epitope, we measured the antibody binding to 55 alanine scanning mutants of $gp120_{JR\text{-}CSF}$ complexed with sCD4 (Table 3). The mutated residues are in all major regions of gp120 and are presumably solvent-exposed.[17] Of those alanine substitutions that do not affect the binding of CD4, mutations of amino acid residues R298, N392, I423, K432, P437 resulted in more than twofold decrease of the m6 binding affinity. The antibody bound to the I423A and

Table 3. Binding of m6 to alanine scanning mutants of $gp120_{JR\text{-}CSF}$ complexed with CD4

Mutant[a]	gp120 domain[b]	Relative affinity[c]	Mutant	gp120 domain	Relative affinity
wt[d]		100	P417A	C4	112
C119A[e]		23	R419A[f]	C4	58
V120A		68	I420A[f]	C4	60
K121A[f]	C1(V1/V2 stem)	76	K421A[f]	C4	73
L122A	C1(V1/V2 stem)	65	Q422A[f]	C4	72
T123A[g]	C1(V1/V2 stem)	64	**I423A**[f]	C4	7
L125A[g]	C1(V1/V2 stem)	107	I424A	C4	118
V127A	C1(V1/V2 stem)	76	N425A[g]	C4	110
T198A	C1(V1/V2 stem)	86	**M426A**[g]	C4	26
S199A[g]	C1(V1/V2 stem)	39	**W427A**[g]	C4	0
V200A[f]	C1(V1/V2 stem)	51	Q428A[g]	C4	104
I201A	C1(V1/V2 stem)	99	E429A[g]	C4	103
T202A	C1(V1/V2 stem)	89	**V430A**[g]	C4	0
Q203A[f]	C2	101	G431A	C4	132
K207A[e]	C2	1	**K432A**[f]	C4	23
S256A[g]	C2	106	M434A[f]	C4	76
T257A[g]	C2	81	**Y435A**[f,g]	C4	23
R298A	C2	40	**P437A**[f]	C4	48
N339A	C3	57	R469A	V5	98
P363A	C3	108	P470A	V5	235
S365A[g]	C3	53	G471A	V5	69
G366A[g]	C3	46	**G472A**	C5	85
G367A[g]	C3	25	**G473A**[g]	C5	6
D368A[g]	C3	34	D474A[g]	C5	286
P369A[g]	C3	95	M475A	C5	87
E370A[g]	C3	64	R476A	C5	118
N386A	C3	99	D477A	C5	83
N392A	V4	39	W479A	C5	187

Mutants with more than a twofold decrease in m6 binding are highlighted in bold face.

[a] The residue numbering scheme is based on the sequence of prototypic HxBc2 gp120 glycoprotein.

[b] C, constant domain; V, variable loop.

[c] Calculated using the formula:

$$[\text{apparent affinity(wildtype)}/\text{apparent affinity(mutant)}] \times 100\%$$

where apparent affinities were calculated as the antibody concentration at 50% maximal binding.

[d] Wild-type JR-CSF gp120.

[e] Residues involved in maintaining the overall structure of gp120.

[f] Residues that exhibit decreased solvent-accessibility in the presence of Fab 17b in the ternary complex.

[g] Residues that exhibit decreased solvent-accessibility in the presence of sCD4 (D1D2) in the ternary complex.

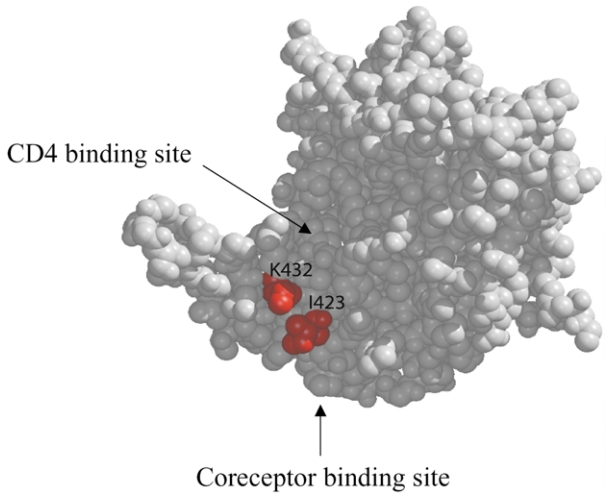

Figure 2. Amino acid residues that are critical for binding of m6 to gp120–CD4 as defined by alanine scanning mutagenesis. The residues I423 and K432 that lead to more than fivefold reduction in binding are indicated on the crystal structure of gp120 complexed with CD4 and 17b; only gp120 is shown.[17]

K432A mutants with more than fivefold lower affinity compared to wild-type gp120, indicating the critical role of these residues for binding (Table 3 and Figure 2). These amino acid residues are critical for Fab X5 binding (R. Darbha *et al.*, unpublished results). They are in a highly conserved region of gp120 located in the bridging sheet of gp120 (Figure 2) that is perhaps the major determinant of the broad neutralizing activity of m6.

Sequence and 3D structure analysis

In an initial attempt to find possible clues for the improved potency of m6 and m9, we sequenced them, compared their sequences to that of X5 (R. Darbha *et al.*, unpublished results) and performed molecular dynamics (MD) simulations based on the crystal structure of X5 provided by X. Ji (R. Darbha *et al.*, unpublished results). m9 has one mutation in the CDR-H3 (D229G), one in the CDR-H2 (S181T) and one near the C terminus of VH (T251N); m6 has one mutation in the CDR-H1 (F157V), one in the framework (Q125R) and, interestingly, two mutations (G114S, G117A) in the linker. It appears that X5 was already almost optimally selected for a high-affinity binding to gp140–sCD4 complexes, and tuning was possible by only a few mutations.

To assess how such single mutations in the variable loops could affect the structure of the antibodies, we performed MD simulations by using the crystal structure of Fab X5 as a template for homologous modeling of the structure of scFv X5, m6 and m9. The results suggest possible differences in the structure of the heavy chain variable loops, especially for m9 compared to scFv X5 (Figure 3(a)), that could lead to the observed differences in binding. For example, for m9 one can speculate that the change in the conformation of the H3, including an increase in flexibility due to the D229G mutation and changes in the orientation of the two residues (W227 and Y233) that are critical for X5 binding, can lead to higher-affinity binding (Figure 3(b)). These considerations demonstrate the conceptual possibility for changes in the antibody structure due to these specific mutations, but how precisely these changes lead to the improved binding affinity remains to be elucidated.

Discussion

The existence of several broadly cross-reactive hmAbs that potently neutralize HIV-1 *in vitro* and can prevent infection *in vivo* suggests their potential as antiretroviral drugs and microbicides.[1–3] Recent clinical trials evaluated the potential of a combination of two of these antibodies, 2G12 and 2F5, for treatment of chronic HIV-1 infections in humans.[4,5] However, although these antibodies clearly can decrease the plasma concentration of HIV-1 in infected humans, it appears that their potency is not sufficient to reduce the HIV-1 load significantly and the long-term virological response is poor.[5] Thus, a further increase in the potency of hmAbs could be critical for their efficacy.

The major finding of our study is that an increase in the potency of one of the most potent, broadly HIV-1-neutralizing hmAb Fabs, X5, is possible, and that this increase does not lead to loss of breadth of neutralization. By using the newly developed approach, SAP, we selected two scFvs, m6 and m9, that have higher neutralization activity and are able to inhibit a broader range of HIV-1 primary isolates compared to scFv X5. The higher potency and increased breadth of neutralization of primary HIV-1 isolates of these antibody fragments is likely due to their improved binding to the HIV-1 Env trimers through a tuning of the 3D structure of some of their variable loops, although further experiments are needed to prove this possibility. The footprint of m6 on gp120 complexed with CD4 includes the highly conserved amino acid residues I423 and K432 that are critical for binding of Fab X5 to gp120–CD4 complexes (R. Darbha *et al.*, unpublished results). Thus, it appears that the major determinant of the broad neutralizing activity of m6 (and probably m9, which is likely to bind to a closely related epitope) is the very conserved nature of the gp120 amino acid residues in its footprint that is located just outside the CD4 binding site on the bridging sheet in gp120.

Recently, a broadly HIV-1-neutralizing hmAb (IgG1 b12) was shown to protect macaques from vaginal challenge with SHIV-1,[3] supporting the

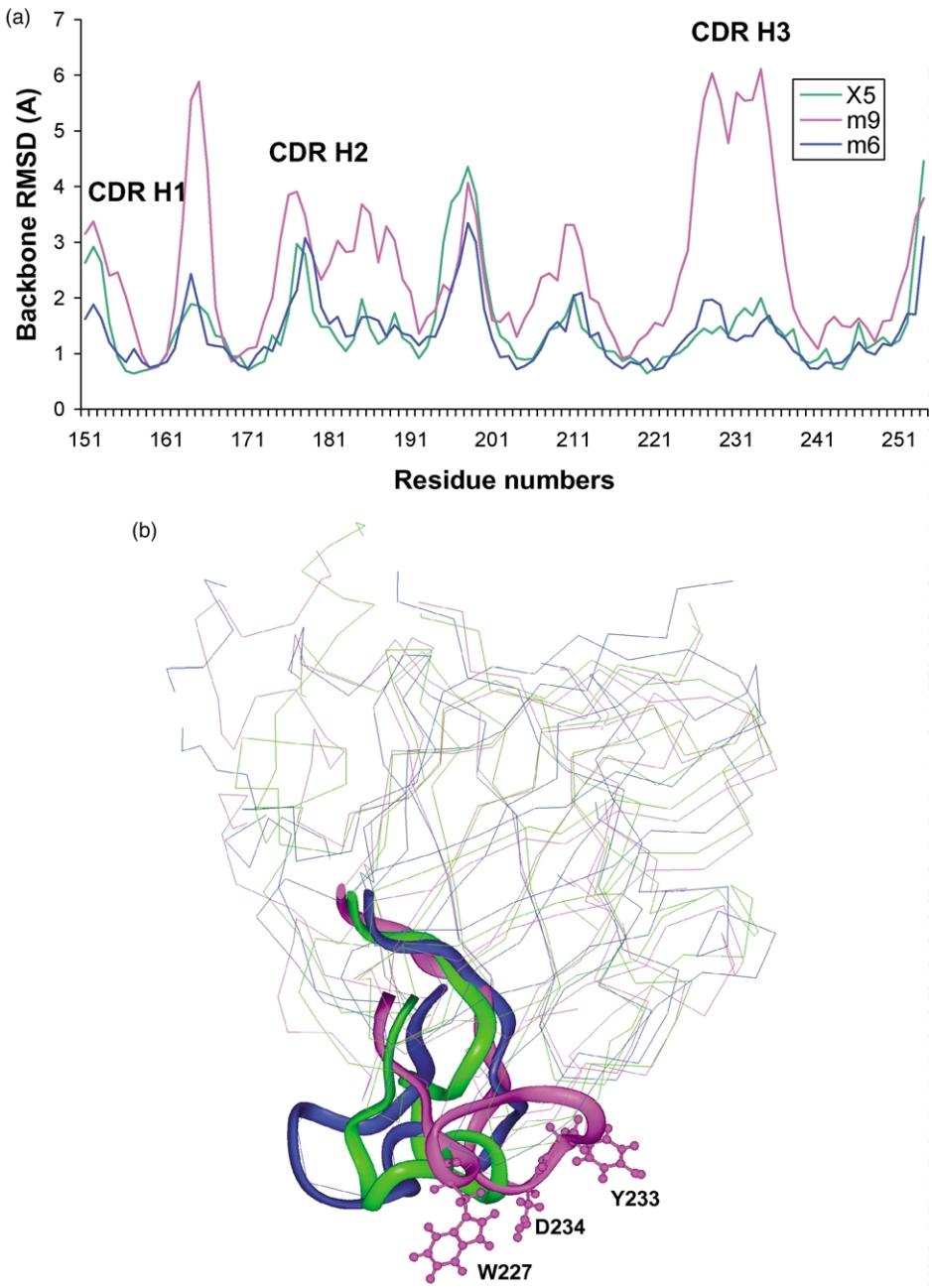

Figure 3. Molecular dynamics simulations of m6, m9 and scFv X5. (a) A plotting diagram of the average per residue root-mean-square deviation (RMSD). Shown is the RMSD for the backbone atoms of two simulations, 250 ps each, calculated by performing the superposition of the corresponding structures at time zero to the conformers obtained during the last 100 ps simulations. (b) Ribbon diagram of a portion of the CDR-H3 of X5, m6 and m9. X5 is shown in green, m6 in blue and m9 in magenta. Surface-exposed residues (W227, Y233 and D234) that are involved in X5 binding are shown by their molecular structures. The m9 mutation (D229G) is between these residues.

concept that antibodies can be used for protection against sexually transmitted virus. Further studies are needed to determine whether m6 and m9 have protective activity *in vivo*. Improvement of their potency could be achieved by constructing fusion proteins with other molecules, e.g. sCD4, hmAbs and toxins, or these antibodies could be used in combination with other drugs and hmAbs. Experiments in animal models may help to determine whether these new antibodies have potential as HIV-1 therapeutics.

Materials and Methods

Cells, viruses, plasmids, soluble CD4 (sCD4), gp120, gp140 and antibodies

293T cells were purchased from ATCC. The CEM cells expressing CCR5 (CEM-CCR5) were a gift from J. Moore (Cornell University, New York, NY). HIV-1 isolates were obtained from the NIH AIDS Research and Reference Reagent Program (ARRRP). Recombinant vaccinia viruses used for the fusion assay have been described.[18] Alanine scanning mutants were obtained by introducing

single alanine substitutions into the pSVIIIexE7pA$_{JR\text{-}CSF}$ background.[19] sCD4 was obtained from the ARRRP. Purified gp120$_{89.6}$, gp140$_{89.6}$ and gp140$_{IIIB}$ were produced by recombinant vaccinia virus (gifts from R. Doms, University of Pennsylvania, Philadelphia, PA and C. Broder, Uniformed Services University of the Health Sciences (USUHS), Bethesda, MD) with a combination of lentil lectin affinity chromatography and size-exclusion chromatography. Recombinant gp120$_{JR\text{-}FL}$ was a gift from A. Schultz & N. Miller (NIAID, Bethesda, MD). Recombinant gp120$_{IIIB}$ was a gift from C. Broder (USUHS, Bethesda, MD). The human monoclonal antibody Fab X5 was produced as described,[8] and the IgG1 X5 was produced as described.[11] The following antibodies were purchased: polyclonal sheep anti-gp120 antibody D7324 (Sigma), Horseradish peroxidase (HRP) conjugated monoclonal mouse anti-M13 antibody (Pharmacia, Uppsala, Sweden), HRP conjugated streptavidin (Zymed Laboratories Inc., San Francisco, CA) and HRP conjugated polyclonal anti-human IgG F(ab′)2 antibodies (Jackson ImmunoResearch, Westgrove, PA). The human monoclonal antibody 17b was a gift from J. Robinson (Tulane University Medical Center, New Orleans, LA).

Construction of the scFv X5 mutant library

The scFv X5 mutant library was generated using standard approaches.[20] The X5 variable regions of the heavy chain (VH) and the light chain (VL) were amplified by PCR using two pairs of primer: VL5Sac (**5′-gTg gCC CAg gCg gCC gAg CTC gTg TTg-3′**) and VL3Link (**5′-gCC ACC TCC GCC TgA ACC gCC TCC ACC AgT TCg TTT gAT CTC CAg TTT gg-3′**) for VL; VH5Link (**5′-ggC ggA ggT ggC TCT ggC ggT ggC ggA TCA gTg CAgCTg CTC gAg CAg TCT gg-3′**) and VH3Spe (**5′-TCg TCg TCg gCA TgT ACT AgT AgA ggA gAC ggT gAC CAg ggT TC-3′**) for VH. ScFv X5 resulted from SOE-PCR of VL and VH. The PCR products were gel-purified, digested with *Sac* I and *Spe* I, and gel-purified again. The purified fragment was then cloned in the phagemid pComb3X linearized by *Sac* I and *Spe* I. To introduce point mutations into the scFv X5 construct, we performed random DNA mutagenesis with the GeneMorph PCR Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions for high-range mutation frequency with slight modification. The first PCR reaction was carried out in a total volume of 50 μl by adding 10 pg of scFv X5 DNA (60 pg of the recombinant phagemid DNA), 20 pmol of primers VL5Sac and VH3Spe each and 2.5 units of mutazyme under the following conditions: an initial denaturation for five minutes at 94 °C followed by 30 cycles at 94 °C for one minute, 55 °C for one minute, 72 °C for one minute and a filling cycle of 72 °C for ten minutes. The first PCR products were gel-purified and 25 ng of purified first PCR products were used as templates in the second PCR amplification under the same conditions as described above. The products from the second PCR amplification were gel-purified and digested with *Sac* I and *Spe* I. The pComb3X containing the Fab X5 insert was digested with the same enzymes and the resulting linearized vector purified by agarose gel electrophoresis. The purified vector DNA was ligated with purified scFv fragments and ligation products electroporated into electrocompetent *E. coli* XL1-blue cells to create an scFv mutant library. Four separated ligations and transformations were pooled to increase the library diversity. The efficiency of this transformation with a total of 320 ng of purified scFv fragments yielded 1.2×10^6 independent transformants. In all, 31 individual clones were selected randomly, and plasmid DNA was prepared and sequenced.

An scFv phage library was prepared from the initial transformations upon infection with the replication defective helper phage M13KO7, as described.[21] The phage titer was determined by dilutions of the exponentially growing *E. coli* XL1-blue cells.

Sequential antigen panning of the scFv mutagenesis library

Phage (5×10^{12} cfu/ml) were preadsorbed on streptavidin-M280-Dynabeads in PBS for one hour at room temperature followed by depletion in an immunotube (Nunc, Denmark) coated with 10 μg/ml of sCD4 for one hour at 37 °C. The depleted phage library was incubated with 50 nM biotinylated oligomeric gp140$_{89.6}$ complexed with sCD4 in solution (molar ratio of gp140$_{89.6}$ to sCD4 = 1:1) for two hours at room temperature with gentle agitation. Phages bound to the biotinylated gp140 were separated from the phage library using streptavidin-M280-Dynabeads and a magnetic separator (Dynal). The beads were washed 20 times with 1 ml of PBS containing 0.1% (v/v) Tween-20 and another 20 times with 1 ml of PBS. Bound phage were eluted by incubation at room temperature for ten minutes with 1 ml of 100 mM triethanolamine (TEA) followed by neutralization with 0.5 ml of 1 M Tris–HCl (pH 7.5). Eluted phage were rescued by infection of *E. coli* TG1 cells and a phage library was prepared for the next round of panning. For the second and third rounds of panning, 10 nM and 2 nM, respectively, of biotinylated oligomeric gp140$_{89.6}$ complexed with sCD4 (1:1 molar ratio) was used as antigen. For the fourth round of panning, 2 nM biotinylated gp140$_{IIIB}$ complexed with sCD4 (1:1 molar ratio) was used as antigen. After the third and fourth rounds of panning, 20 individual clones were screened by phage ELISA for binding to gp140$_{89.6}$, gp120$_{JRFL}$, gp140$_{IIIB}$ and their complexes with sCD4 by the following procedure. The phage supernatants from individual clones used in phage ELISA were prepared as described.[22]

Phage ELISA

Phage ELISA was performed by using 96 well NuncImmunoTM MaxisorpTM surface plates (Nalge Nunc International, Denmark), which were coated overnight at 4 °C with 100 μl of gp120/gp140 (1 μg/ml in sodium bicarbonate buffer, pH 8.3) or gp120/140–sCD4 complexes (100 μg/ml gp120/gp140 in PBS were premixed with an equal volume of 100 μg/ml of sCD4). After incubation at room temperature for 30 minutes, the mixture was diluted to 1 μg/ml in PBS, blocked in 100 μl of 4% (w/v) non-fat dry milk in PBS for one hour at 37 °C. After four washes with WB (0.05% (v/v) Tween-20 in PBS), wells were incubated with 100 μl of phage supernatant for two hours at 37 °C. Bound phages were detected by using HRP conjugated anti-M13 monoclonal antibody (Pharmacia) with incubation for one hour at 37 °C and revealed by adding ready-to-use ABTS substrate (Pharmacia). Color development was performed at room temperature for 15 minutes and monitored at 405 nm.

Preparation of soluble scFv fragments

The pComb3X phagemid containing m6 and m9 scFv

genes were prepared and transformed to *E. coli* Top 10. Soluble scFvs were expressed as described[23] and His_6-tagged scFvs purified by immobilized metal ion affinity chromatography (IMAC) using Ni-NTA resin according to manufacturer's protocols.

Biotinylation of soluble scFvs

From 0.1 mg/ml to 1.0 mg/ml of affinity-purified scFvs in PBS buffer was mixed with 0.1 vol. 1 M $NaHCO_3$ (pH 8.6) and a 15–20-fold molar excess of biotinylation reagent (2 mg/ml in DMSO) added at room temperature for 30 minutes. The reaction was stopped by adding 0.05 vol. 2 M glycine. Free biotinylation reagent was removed by centrifugation using Microcon (YM-10) (Millipore).

Generation of recombinant HIV-1 virions and expression of recombinant gp120

To produce recombinant virions, 293T cells grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with penicillin, streptomycin, L-glutamine, and 10% (v/v) fetal bovine serum (FBS) were transiently transfected with wild-type or mutant $pSVIIIexE7pA^{-}_{JR\text{-}CSF}$ plasmids (2 μg) along with the luciferase reporter plasmid pNL4.3.Luc.$R^{-}E^{-}$ (4 μg) (obtained from the NIH ARRRP and contributed by Nathaniel Landau)[24] by using FuGENE6 transfection reagent (Roche). At 24 hours post-transfection, the culture supernatant was replaced with serum-free medium, and incubation was continued for another 24 hours. Cell culture supernatants containing pseudovirions were harvested subsequently. Recombinant virions in cell culture supernatants were either lysed by the addition of detergent to the harvested culture supernatants and used in determination of antibody apparent affinity or stored at −80 °C and used in neutralization assays.

Enzyme-linked immunosorbent assays (ELISAs)

ELISA was performed by using 96 well Nunc-ImmunoTM MaxisorpTM surface plates. Coating of antigen and washing and blocking steps were the same as described for phage ELISA. For scFv binding assay, microplate wells were incubated with 100 μl of twofold serially diluted biotinylated soluble scFv for two hours at 37 °C. After four washes with WB, 100 μl of a 1:2500 (v/v) dilution of HRP-streptavidin was added and incubated for one hour at 37 °C. Following four washes with WB, the assay was developed at 37 °C for 15–30 minutes with ready-to-use ABTS substrate and monitored at 405 nm. For competition ELISA, 50 μl of twofold serially diluted competing scFv hmAbs (m6, m9 or scFv X5) were added to the blocked and washed wells, immediately followed by addition of 50 μl of Fab or IgG hmAbs (X5, IgG 17b, IgG b12) previously determined to result in an ELISA signal that was 50–75% of maximum without competitor. After incubation for two hours at 37 °C, the wells were washed as above, probed with an HRP conjugated anti-human IgG $F(ab')_2$ conjugate (Pierce) diluted 1:2500 (v/v) in PBS containing 2% (w/v) non-fat dry milk and detected as described above.

To measure the apparent affinity of m6 for recombinant gp120 with various alanine substitutions, capture ELISAs were performed. Microtiter plate wells (flat-bottom; Costar type 3690; Corning Inc.) were coated overnight at 4 °C with anti-gp120 antibody D7324 at a concentration of 5 μg/ml (250 ng/well; diluted in PBS). Subsequent incubation steps were performed at room temperature. Coated plates were washed twice with WB, blocked for one hour with PBS supplemented with 3% (w/v) bovine serum albumin, and then incubated for two to four hours with cell culture supernatants that had been diluted 1:3 (v/v) in PBS-B-T (PBS containing 1% bovine serum albumin and 0.02% Tween). Plates were washed with WB (ten times) and then incubated with biotinylated m6 serially diluted in PBS-B-T (starting at a concentration of 2 μg/ml). Human IgG purified from pooled plasma obtained from healthy asymptomatic seropositive individuals (1 μg/ml; diluted in PBS-B-T) was used as a control to ensure that similar amounts of envelope protein were captured. After plates were washed as described above, HRP-streptavidin conjugate was added (diluted 1:1000 (v/v) in PBS-B-T), and incubation was continued for another hour. Plates were washed again and then incubated with tetramethylbenzidine substrate. The color reaction was stopped by adding 2 M sulfuric acid, and absorbances were measured at 450 nm. Apparent binding affinities were calculated as the antibody concentration at half-maximal binding; percentage changes in affinity relative to that of the wild-type were expressed as relative affinity, which is:

$$(\text{apparent affinity of the wild type}/\text{apparent affinity of the mutant}) \times 100$$

Soluble CD4-induced cell–cell fusion assay

Fusion between 293 cells, expressing CXCR4 after infection with recombinant vaccinia viruses (DM1107), and TF228 cells, expressing HIV-1IIIB Env, induced by soluble CD4 was measured by the β-galactosidase assay. The 293 cells expressing CXCR4 were mixed with sCD4 (5 μg/ml), m6, m9, X5 or IgG b12 (0.01, 0.1, 1 μg/ml) and TF228 cells. In a control experiment, no antibody was added. Fusion was allowed to proceed for three hours at 37 °C and quantified by a colorimetric assay that measures the optical absorbance at 595 nm (A_{595}).

HIV-1 neutralization assays

Three HIV-1 neutralization assays were used in this study. The first is based on infection of PBMCs with infectious molecular clones and measurement of p24 seven days after infection as described.[8] In the second assay format, single-round infectious molecular clones, produced by envelope complementation, were used. The degree of virus neutralization by antibody was achieved by measuring luciferase activity. Briefly, 2×10^4 U87.CD4.CCR5.CXCR4 cells (obtained through ARRRP from H. Deng and D. Littman[25]) in 100 μl of medium (DMEM containing 15% FBS, 1 μg of puromycin/ml, 300 μg of G418/ml, glutamine, and penicillin–streptomycin) were added to microplate wells (96 well, flat-bottom; Corning Inc., Corning, N.Y.) and incubated for 24 hours at 37 °C in 5% (v/v) CO_2. One hundred microliters of medium containing virus was mixed with various amounts of antibody, incubated for one hour at 37 °C, added to the cells, and incubated for a further three days. The wells were aspirated and washed once with PBS, and 60 μl of luciferase cell culture lysis reagent (Promega, Madison, WI) was added. The wells were scraped and the lysate was mixed by

pipetting, 50 μl were transferred to a round-bottom plate (Corning), and the plate was centrifuged at 1800 *g* for ten minutes at 4 °C. Twenty microliters were transferred to an opaque assay plate (Corning), and the luciferase activity was measured on a luminometer (EG&G Berthold LB 96V; Perkin–Elmer, Gaithersburg, MD) by using luciferase assay reagent (Promega).

The third HIV-1 neutralization assay is based on the use of infectious virus and a reporter gene cell-line JC-53-BL. Primary HIV-1 isolates were either isolated from Institutional Review Board-approved CDC Studies or obtained from the NIH Research and Reference Reagent Program through the WHO collaborative network. The detailed characteristics of various isolates, including subtype determination based on the envelope region and coreceptor usage using GHOST cell lines has been described.[26–28] Viral stocks were generated by infection of CD8-depleted normal human PBMC as described.[26,27] Viral stocks were filtered through 0.22 μm filters, aliquoted and maintained at −70 °C. A reporter gene-based viral replication assay was used to read out the viral replication.[29] Briefly, JC53-BL, an HIV-1 indicator cell line derived from HeLa cells that express high levels of CD4, CXCR4 and CCR5, contains reporter cassettes for luciferase and β-galactosidase both driven by the HIV-1 LTR (a kind gift from Tranzyme Inc., Birmingham, AL). These cassettes allow detection of HIV-1 infection (tat production) by measuring either luciferase activity with a luminometer or by counting blue foci after staining the cells with X-gal. The JC53-BL cells are maintained in c-DMEM, which is DMEM supplemented with 10% fetal calf serum (Hyclone), 2 mM glutamine (Gibco), 100 units/ml of penicillin G and 100 μg/ml of streptomycin (Gibco). The viral titers were determined by adding serial dilutions of the virus stocks in C-DMEM containing 40 μg/ml of DEAE-dextran to 20,000 JC53-BL cells per well, in duplicate, in 96 well plates. Following 48 hours incubation at 37 °C in a 5% CO_2 incubator, the cells were fixed and stained. Blue foci were counted using a standard light microscope and the titers are expressed as infectious units or blue foci units per ml. From the infectious unit data, multiplicity of infection (MOI) values are determined for the inhibition assays.

The neutralizing activities of the antibodies in this assay were determined as follows. The JC53-BL cells were removed from T-150 flasks using 0.017 M PBS, 0.1 mM EDTA at a pH of 7.4 approximately 18 hours prior to starting the inhibition assays and were plated at a density of 20,000 cells per well in white, 96 well plates in 50 μl of C-DMEM. Viral stocks (MOI range of 0.009 to 0.65) were pre-incubated with different concentrations of the mAbs (final concentrations of 100–0.05 μg/ml) for one hour, prior to addition to the cells in medium containing 40 μg/ml of DEAE-dextran to give a final volume of 200 μl per well. The plates were incubated in a 37 °C, 5% CO_2 incubator for 48 hours, and luciferase activity was measured using the Steady-Glo Luciferase Assay System (Promega, Madison, WI) following the manufacturer's lysis protocol. The light intensity was measured using a Tecan luminometer with Magellan software (Tecan, Research Triangle Park, NC) and values calculated as relative light units (RLU). Percentage inhibition was calculated by the following formula:

$$1 - (\text{average RLU of mAb-containing wells} / \text{average RLU of virus-only wells}) \times 100$$

All assays were performed in triplicates.

Molecular dynamics (MD) simulations

The initial three-dimensional structure models for the MD simulations were constructed by using the X-ray crystal structure of Fab X5 (R. Darbha *et al*., unpublished results) as template to simulate the VH and VL domains of scFv X5. The linker $(G_4S)_3$ between VH and VL domains was modeled by the loop generation function of the Homology module of the InsightII (Molecular Simulation Inc., San Diego, CA). The other two scFv antibodies m6 and m9 were built by mutating *in silico* (using the Biopolymer module) the residues G114S, G117A (linker region), Q125 R (framework region) and F157V (CDR-H1) for m6; S181T (CDR-H2), D229G (CDR-H3) and T251N (near the C terminus) for m9. The MD simulations were performed on SGI Origin2000 by the Discover module of InsightII. Each of the structures was submitted to 300 steps of energy minimization, then 50 ps equilibration at 300 K and finally 200 ps MD simulations, collecting one conformation in every 0.5 ps. To take into account the possibility of statistical errors and to test the reproducibility of the results, the MD dynamics simulations were run twice using the conditions described above.

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